A Previously Unreported Function of β_1B Integrin Isoform in Caspase-8-Dependent Integrin-Mediated Keratinocyte Death

Roberta Lotti[1,3], Alessandra Marconi[1,3], Francesca Truzzi[1], Katiuscia Dallaglio[1], Claudia Gemelli[2], Riccardo G. Borroni[1], Elisabetta Palazzo[1] and Carlo Pincelli[1]

Integrins regulate adhesive cell–matrix interactions and mediate survival signals. On the other hand, unligated or free cytoplasmic fragments of integrins induce apoptosis in many cell types (integrin-mediated death). We have previously shown that β_1 integrin expression protects keratinocyte stem cells from anoikis, whereas the role of the β_1B integrin isoform has not been clarified. In this study we report that suspended keratinocytes undergo apoptosis through the activation of caspase-8, independently of the Fas/Fas ligand system. Indeed, anti-β_1 integrin-neutralizing antibodies induced apoptosis in short hairpin RNA Fas-associated death domain-treated cells. Moreover, before and during suspension, caspase-8 directly associated with β_1 integrin, which in turn internalized and progressively degraded, shedding the cytoplasmic domain. β_1B was expressed only in the cytoplasm in a perinuclear manner and remained unaltered during suspension. At 24 hours, as β_1A was located close to the nucleus, β_1B colocalized with β_1A and coimmunoprecipitated with caspase-8. Caspase-8 was activated earlier in β_1B integrin-transfected keratinocytes, and these cells underwent a higher rate of apoptosis than mock cells. In contrast, caspase-8 was not activated in small interfering RNA (siRNA) β_1B-transfected cells. These results indicate that when β_1A is unligated, β_1B is responsible for "integrin-mediated death" in human keratinocytes.

Journal of Investigative Dermatology (2010) **130,** 2569–2577; doi:10.1038/jid.2010.195; published online 8 July 2010

INTRODUCTION

β_1 Integrin is abundantly expressed in keratinocytes and is critical for its adhesion to type IV collagen (Fleischmajer *et al.*, 1998; Tiberio *et al.*, 2002). A β_1B variant has also been isolated, in which a unique 12-amino-acid sequence replaces the last 21 COOH-terminal amino acids of wild-type β_1 integrin (Altruda *et al.*, 1990). Although β_1B integrin is expressed not only in keratinocytes, but also in liver and in skeletal and cardiac muscles (Balzac *et al.*, 1993), its functional properties remain largely unknown. β_1 Integrins mediate binding to the extracellular matrix and transduce an intracellular signal promoting cell survival in many tissues and cells, including keratinocytes (Howlett *et al.*, 1995; Marconi *et al.*, 2004). Cells that are denied adhesion because of integrin ligand deprivation undergo a form of cell death named anoikis (Frisch and Francis, 1994). Although anoikis occurs as the consequence of loss of integrin-mediated attachment, a form of integrin-mediated death (IMD) has been proposed, by which integrins in the antagonized or unligated state can promote apoptosis independently of cell detachment (Brassard *et al.*, 1999; Stupack *et al.*, 2001). In most instances, anoikis process involves the extrinsic apoptotic pathway that is typically triggered by death receptor ligands, which in turn recruit the adaptor protein Fas-associated death domain (FADD). The death receptor-bound FADD associates with caspase-8 in the death-inducing signaling complex, leading to caspase auto-activation (Muzio *et al.*, 1998; Marconi *et al.*, 2004) (Supplementary Figure S1 online). In IMD, integrins seem to cluster with caspase-8 without the involvement of the death-inducing signaling complex (Stupack *et al.*, 2001), although the actual mechanisms underlying this process remain to be clarified. In this study, we show that β_1B integrin clusters with unligated wild-type β_1 integrin and (pro-)caspase-8 to induce IMD in human keratinocytes, providing evidence for a previously unidentified role of this integrin isoform.

[1]*Institute of Dermatology, School of Biosciences and Biotechnologies, University of Modena and Reggio Emilia, Modena, Italy and* [2]*Department of Biomedical Sciences, School of Biosciences and Biotechnologies, University of Modena and Reggio Emilia, Modena, Italy*

[3]*These two authors contributed equally to this work.*

Correspondence: Carlo Pincelli, Institute of Dermatology, School of Biosciences and Biotechnologies, University of Modena and Reggio Emilia, Via del Pozzo, 71, Modena 4100, Italy. E-mail: carlo.pincelli@unimore.it

Abbreviations: FasL, Fas ligand; IMD, integrin-mediated death; SCC, squamous cell carcinoma; siRNA, small interfering RNA; shFADD, short-hairpin-RNA-Fas-associated death domain.

Received 9 November 2009; revised 5 May 2010; accepted 13 May 2010; published online 8 July 2010

RESULTS

Death receptors are not involved in anoikis

To test the role of β_1 integrin and caspase-8 during anoikis, cells were suspended and treated with a neutralizing anti-β_1 integrin antibody. We show that suspension induces

© 2010 The Society for Investigative Dermatology

keratinocyte apoptosis that is significantly augmented by blocking β_1 integrin, indicating that this integrin has a role in keratinocyte anoikis (Supplementary Figure S1a online) (Marconi *et al.*, 2004). We also show that caspase-8 is activated early in keratinocyte anoikis (Supplementary Figure S1b online), suggesting the involvement of death receptors in this process. To evaluate whether death receptors are actually responsible for caspase-8 activation and anoikis in our system, we evaluated the modulation of Fas/Fas ligand (FasL) in suspended keratinocytes and after treatment with anti-β_1 integrin. During a 0- to18-hour time course, no upregulation of either Fas (Figure 1a) or FasL (Figure 1b) was detected in both suspended and anti-β_1 integrin-treated keratinocytes. In addition, no FasL was released by suspended keratinocytes up to 3 hours (Figure 1c). To definitely rule out the involvement of death receptors in keratinocyte anoikis, we retrovirally infected HaCaT keratinocytes with short hairpin RNA Fas associated death-domain (shFADD). Western blot analysis showed reduced levels of FADD in short hairpin RNA FADD-infected keratinocytes when compared with

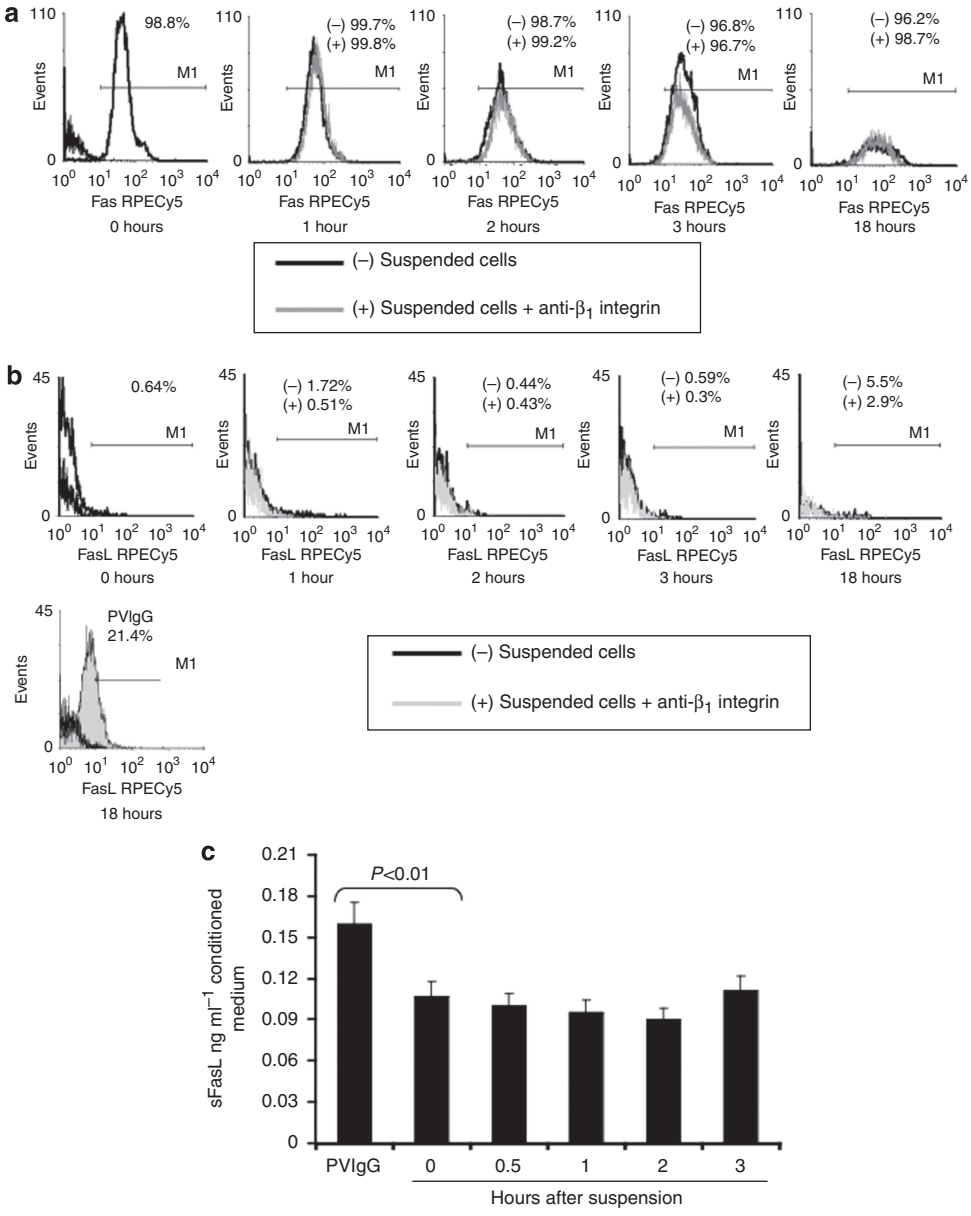

Figure 1. Fas/Fas ligand (FasL) modulation in keratinocyte anoikis. (**a**) Fas expression. Keratinocytes were suspended in polypropylene tubes in the presence or absence of anti-β_1 integrin-neutralizing antibody. Cells were then incubated with anti-Fas antibody and analyzed by flow cytometry. (**b**) FasL expression. Keratinocytes were treated as before, incubated with anti-FasL antibody, and analyzed by flow cytometry. Pemphigus vulgaris IgG (PVIgG) was used as a positive control stimulus for FasL (Arredondo *et al.*, 2005). (**c**) Soluble FasL (sFasL) release. Keratinocytes were kept in suspension in polypropylene tubes for different time points and culture media were collected. sFasL in culture media was analyzed by ELISA. PVIgG was used as a positive control stimulus for FasL.

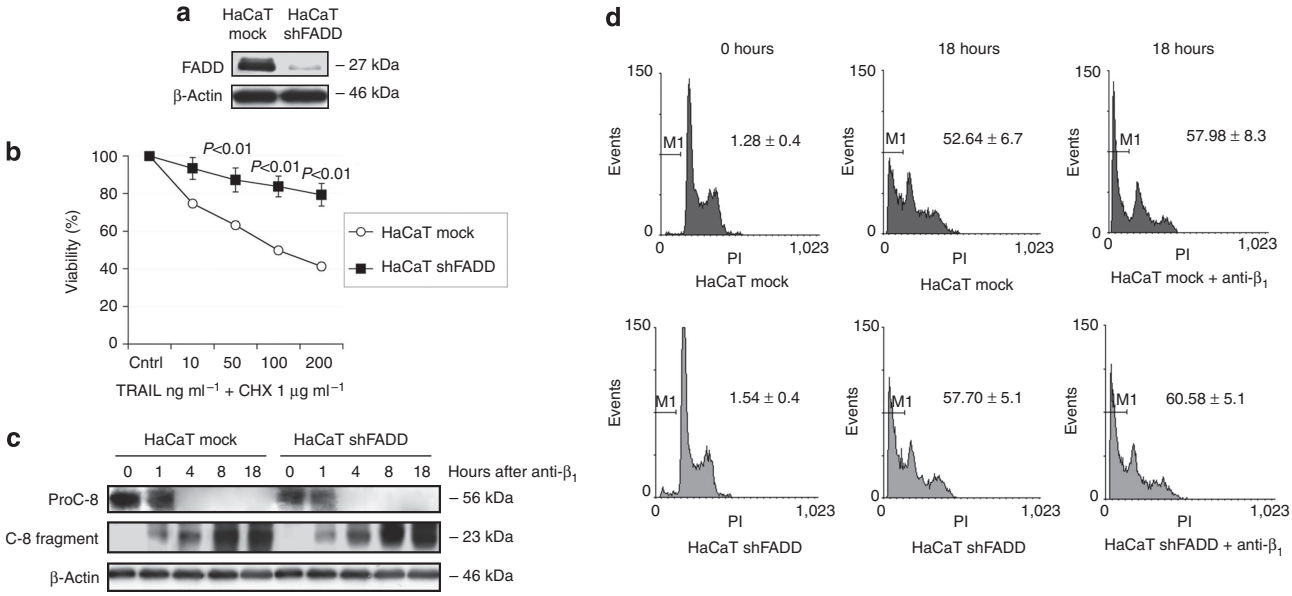

Figure 2. Fas-associated death domain (FADD) protein is not required for keratinocyte anoikis. (**a**) FADD protein expression in infected HaCaT keratinocytes. Proliferating HaCaT cells were retrovirally infected with FADD short hairpin RNA (shRNA; HaCaT shFADD) or with control shRNA (HaCaT mock). Cells were selected in the presence of puromycin and lysed. Lysates were run on SDS-acrylamide gel and blotted onto nitrocellulose membrane. Blot was incubated against anti-FADD antibody. β-Actin was used as loading control. (**b**) shFADD prevents tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis in keratinocytes. Infected HaCaT cells were treated with increasing doses of TRAIL and viability was analyzed by MTT assay after 24 hours. (**c**) Caspase-8 activation in shFADD cells. Retrovirally infected HaCaT cells were suspended and treated with anti-β_1 integrin-neutralizing antibody. Cells were lysed at different time points, and lysates were immunoblotted with anti-caspase-8 antibody. β-Actin was used as loading control. (**d**) shFADD cells show the same percentage of sub-G1 peak positive cells. Mock and shFADD keratinocytes were kept in suspension in polypropylene tubes in the presence or absence of anti-β_1 integrin antibody. After 18 hours, cells were stained in propidium iodide (PI) solution. Sub-G1 peak positive cells (M1) were analyzed by flow cytometry. Data represent the mean from three independent experiments.

mock cells (Figure 2a). We first tested the capability of shFADD to prevent apoptosis. As expected, tumor necrosis factor-related apoptosis-inducing ligand induced apoptosis in mock but not in shFADD keratinocytes (Figure 2b). In contrast, caspase-8 was activated in shFADD as well as in mock keratinocytes treated with anti-β_1 integrin, as shown by the appearance of the active fragment (23 kD; Figure 2c). Furthermore, both shFADD and mock keratinocytes showed the same percentage of sub-G1 peak positive cells in the presence or absence of anti-β_1 integrin. At 18 hours, although keratinocytes reached high degree of anoikis and caspase-8 was fully activated, blocking FADD did not prevent cell death in keratinocytes (Figure 2d).

Direct interaction of β_1 integrin and caspase-8 in adherent and suspended cells

If keratinocytes undergo anoikis without the involvement of FADD, we reasoned that there must be a direct interaction between β_1 integrin and caspase-8. To determine whether caspase-8 and β_1 integrin interact in keratinocytes, adherent cells were double stained with anti-β_1 integrin and anti-caspase-8 antibodies. Subconfluent keratinocytes expressed β_1 integrin in both the membrane and cytoplasm, whereas caspase-8 showed a diffuse cytosolic localization. Double staining revealed that β_1 integrin and caspase-8 colocalize in the cytoplasm (Figure 3a). Consequently, we investigated the interaction between caspase-8 and β_1 integrin in keratinocyte anoikis. Western blotting on lysates from suspended keratinocytes showed the appearance of caspase-8 active fragment (18 kDa), starting at 1 hour (Figure 3b). The same lysates were immunoprecipitated with anti-β_1 integrin antibody. Procaspase-8 and β_1 integrin coimmunoprecipitated at 0 hours, whereas procaspase-8 progressively decreased, indicating activation, up to 24 hours. Furthermore, suspension affected β_1 integrin conformation starting at 1 hour, and precipitated protein decreased at 6 hours, eventually disappearing at 24 hours. FADD did not immunoprecipitate with β_1 integrin–procaspase-8 complex, further confirming that death receptors are not involved in keratinocyte anoikis (Figure 3c). Taken together, these results clearly indicate that β_1 integrin and caspase-8 directly interact, and that β_1 integrin is altered during anoikis.

β_1 Integrin is internalized and degraded during suspension

As β_1 integrin is progressively altered during keratinocyte suspension, we wanted to investigate the fate and the function of β_1 integrin in this system. To this end, we incubated keratinocytes with neutralizing anti-β_1 integrin antibody and performed anoikis assay. Confocal images showed that β_1 integrin was partially internalized after 30 minutes and almost completely at 2 hours (Figure 4a). Indeed, as suspension proceeded, cell-to-cell contacts progressively decreased along with the internalization of integrin, in agreement with the observation that cells

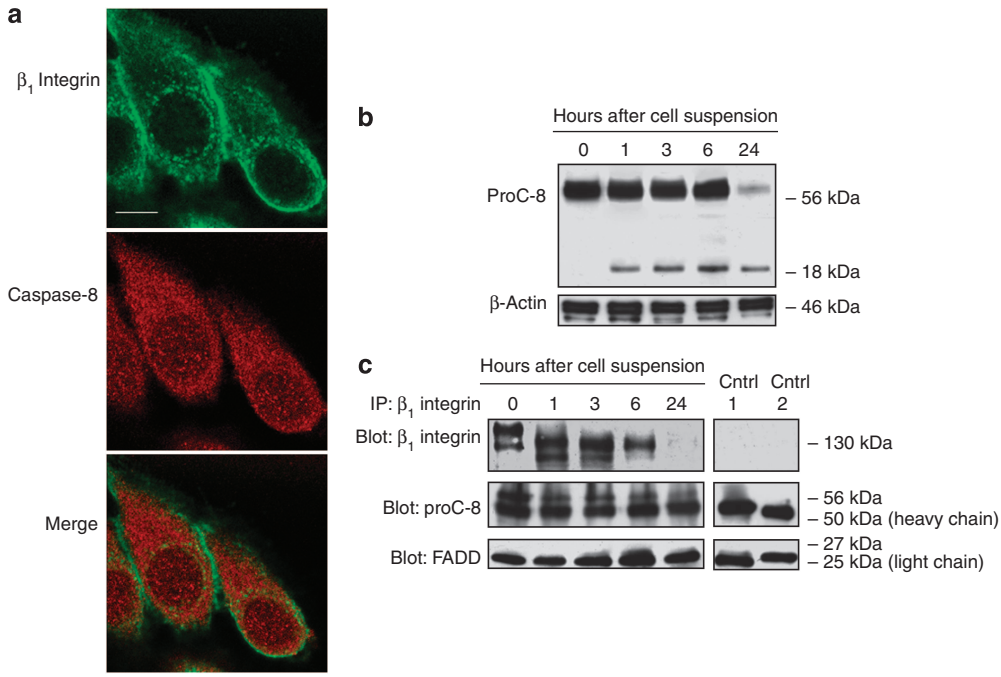

Figure 3. β_1 Integrin associates with caspase-8. (**a**) β_1 Integrin colocalizes with caspase-8. Subconfluent normal human keratinocytes were fixed in 4% paraformaldehyde (PFA). Cells were immunostained with anti-β_1 integrin and anti-procaspase-8 antibodies. Bottom panel represents the merge. Cells were analyzed by confocal microscopy. Bar = 15 μm. (**b**) Early activation of caspase-8 during anoikis. Normal human keratinocytes were trypsinized (0 hours) and kept in suspension in polypropylene tubes for different time points. At the end of single time point, cells were lysed and lysates were immunoblotted with anti-procaspase-8 antibody. β-Actin was used as loading control. (**c**) β_1 Integrin immunoprecipitates with procaspase-8. Keratinocytes were treated as in **b**. Lysates were immunoprecipitated with anti-β_1 integrin antibody. Immunoprecipitates were blotted with anti-β_1 integrin, anti-procaspase-8, and anti-FADD antibodies. Control 1, immunoprecipitate with anti-β_1 integrin antibody without lysates; control 2, immunoprecipitate with a non-keratinocyte antigen antibody (anti-vimentin). Control samples were run simultaneously in a different gel.

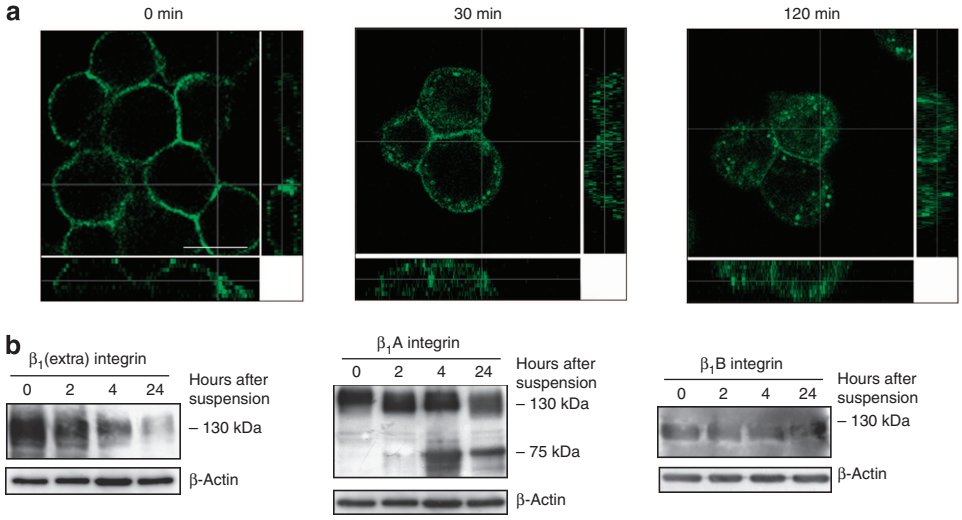

Figure 4. β_1 Integrin is internalized and degraded during keratinocyte suspension. (**a**) β_1 Integrin internalization. Keratinocytes were incubated with the neutralizing anti-β_1 integrin antibody for 20 minutes at 4 °C. Cells were then suspended for different time points, fixed in 4% paraformaldehyde (PFA), and analyzed by confocal microscopy. Images outside the *x*–*y* planes represent the *z*-line projection of the cross-identified area. Bar = 8.5 μm. (**b**) β_1 Integrin degradation. Keratinocytes were suspended in polypropylene tubes and lysed at different time points. Lysates were probed against β_1 integrin, using an antibody directed to the extracellular portion. The same lysates were blotted with anti-β_1A and β_1B integrin antisera. β-Actin was used as loading control.

detached from each other are more prone to this kind of cell death (Frisch and Francis, 1994). Moreover, in suspended cells, the extracellular portion of β_1 integrin was progressively degraded up to 24 hours, as shown by the use of an antibody directed against this integrin domain (Figure 4b). When suspended keratinocytes were blotted using an antibody raised against the full-length β_1A integrin, a band of approximately 75 kD was detected between 2 and 24 hours

(Figure 4b), representing the cytoplasmic fragment of β_1A integrin, likely derived from the degradation of the extracellular domain. β_1B differs from wild-type β_1 integrin (β_1A) in the cytoplasmic domain (Altruda *et al.*, 1990) and is mainly detected within the endoplasmic reticulum (Balzac *et al.*, 1993; Kee *et al.*, 2000). In agreement with previous works, we show that β_1A variant was mostly expressed at the cell membrane. In contrast, we detected β_1B form at the cytoplasmic level in a perinuclear manner (Supplementary Figure S2a online). Given the difference in the cytoplasmic tails, we used specific antibodies recognizing the different β_1 integrin domains. Although both β_1A and β_1B were expressed in human keratinocytes, the content of β_1A protein was markedly higher than that of β_1B integrin (Supplementary Figure S2b online). The role of this integrin form in keratinocytes is not fully understood. It has been proposed that β_1B acts as a dominant-negative regulator of cell adhesion, by interfering with focal contact formation, thus decreasing adhesiveness (Retta *et al.*, 1998). To better understand the role of this β_1 integrin variant, we suspended human keratinocytes and blotted lysates using an antibody raised against β_1B integrin isoform. Unlike β_1A, no protein modifications of β_1B were observed up to 24 hours (Figure 4b), indicating that no degradation of this variant occurs during suspension.

β_1A and β_1B integrins cluster and immunoprecipitate with caspase-8 during suspension

Confocal images of cells at 0-hour suspension show that β_1A expression was strictly localized at the cell membrane, whereas active caspase-8 was still undetectable. At 1 hour, caspase-8 became activated, whereas β_1A began to internalize. After 24 hours of suspension, caspase-8 was clearly detected with a cytoplasmic punctate pattern. At this time, β_1A integrin was also observed in perinuclear location (Figure 5a). On the other hand, at 0 hours, β_1B was predominantly expressed at the perinuclear level. This expression pattern remained unchanged up to 24 hours, when caspase-8 was fully activated (Figure 5b). These results suggest that during keratinocyte suspension, β_1A integrin internalizes and locates close to the nucleus, likely colocalizing with β_1B. As β_1A and β_1B integrins colocalize with caspase-8 at the cytoplasmic level, we analyzed whether caspase-8 coimmunoprecipitates with either of these integrin isoforms. At 0 hours, we observed a weak band, indicating that a small amount of procaspase-8 bound to both β_1

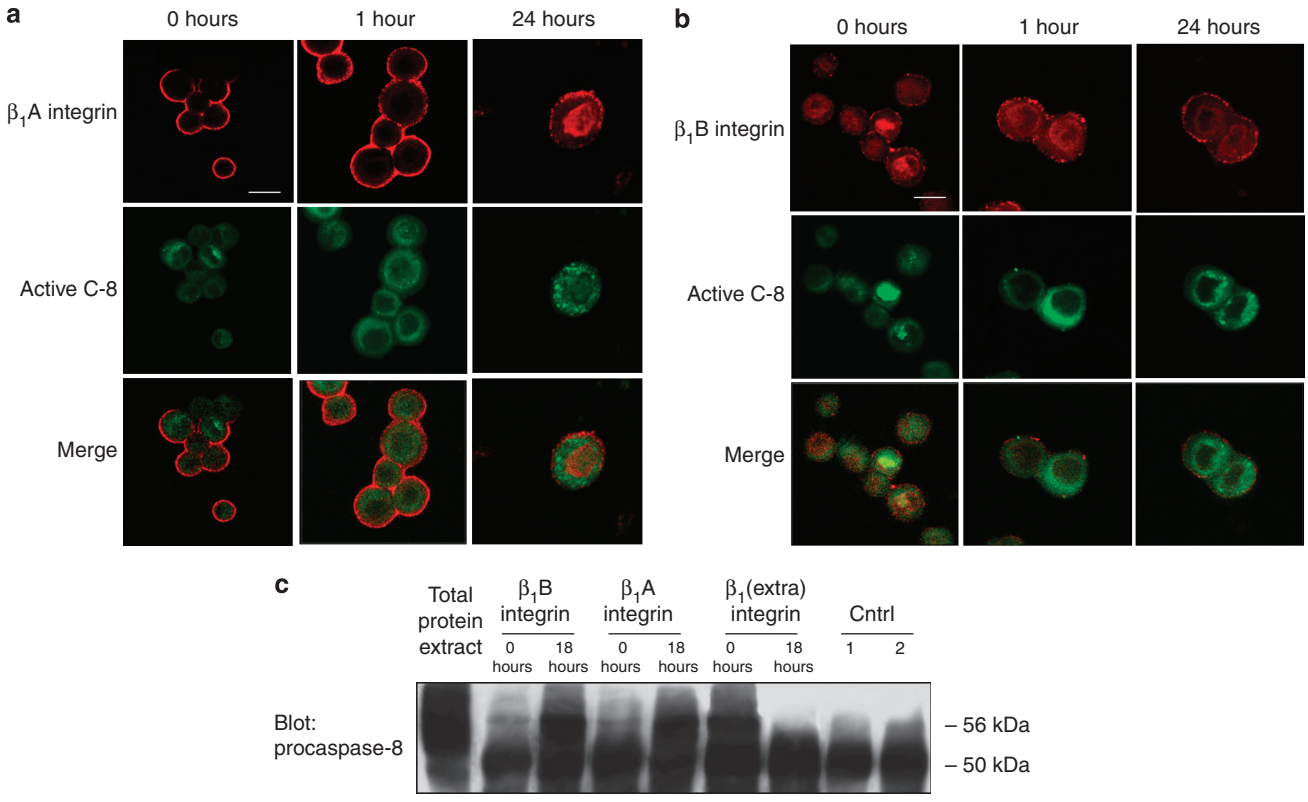

Figure 5. β_1 Integrin variants immunoprecipitate with caspase-8 during suspension. (**a, b**) β_1A and β_1B integrin localization during caspase-8 activation. Keratinocytes were suspended in polypropylene tubes and fixed in 4% paraformaldehyde (PFA) at different time points. Cells were spun and immunostained at room temperature with (**a**) anti-β_1A, (**b**) anti-β_1B integrin, and (**a, b**) antisera and anti-active caspase-8 antibody. Cells were analyzed by confocal microscopy. (**a**) Bar = 21 μm. (**b**) Bar = 17 μm. (**c**) β_1 Integrin variants immunoprecipitate with procaspase-8. Keratinocytes were suspended in polypropylene tubes and lysed after 18 hours of suspension. Lysates were alternatively immunoprecipitated with anti-β_1B integrin, anti-β_1A integrin, or anti-extracellular domain of β_1 integrin antibodies. Immunoprecipitates were blotted with anti-procaspase-8. Control 1, immunoprecipitate without lysates; control 2, immunoprecipitate with total rabbit serum.

integrin variants. After 18 hours of suspension, binding of β_1A or β_1B to caspase-8 was markedly enhanced. As negative controls, no precipitates were observed when lysates were omitted or when primary antibody was replaced by total rabbit serum (Figure 5c).

Proapoptotic role of β_1B integrin

To definitely assess the proapoptotic role of β_1B integrin, keratinocytes were transfected with a full-length β_1B-expressing vector. β_1B was upregulated at 24 hours (Figure 6a), and it remained overexpressed until 48 hours, both at the mRNA and the protein level (Figure 6a and b). β_1B integrin expression was strikingly increased at the intracellular level in transfected cells up to 48 hours (Figure 6c). Moreover, keratinocytes that expressed high levels of β_1B integrin spontaneously detached from the culture dishes when compared with mock cells (Figure 6e) and with keratinocytes transfected with a full-length β_1A-expressing vector, in which adhesion and morphology were not affected (Figure 6d and e). Before inducing suspension, keratinocytes underwent cell

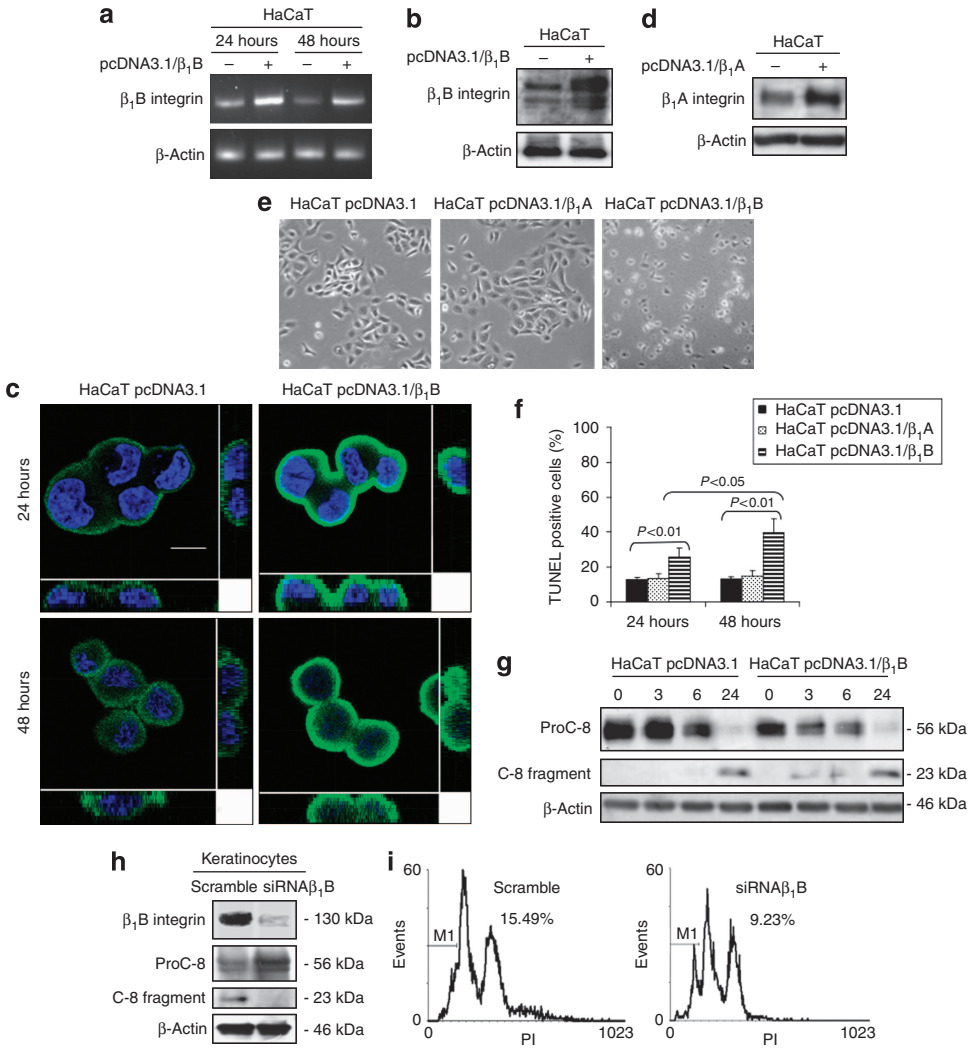

Figure 6. β_1B integrin is proapoptotic in keratinocytes. (**a**) HaCaT cells were transfected with a full-length β_1B integrin-containing vector (pcDNA3.1/β_1B) or with the empty vector (pcDNA3.1). mRNA was extracted from cells after 24 and 48 hours of transfection, and β_1B integrin expression was analyzed by reverse transcriptase- PCR (RT-PCR). β-Actin was used as loading control. (**b**) HaCaT cells were transfected as in **a**. Cells were lysed and protein extract were blotted with anti-β_1B integrin antibody. β-Actin was the loading control. (**c**) HaCaT cells were transfected as before. Keratinocytes were fixed in 4% paraformaldehyde (PFA) after 24 and 48 hours of transfection. Cells were spun and stained with anti-β_1 integrin antibody and analyzed at confocal microscopy. Images outside the *x–y* planes represent the *z*-line projection of the cross-identified area. Bar = 12 μm. (**d**) HaCaT cells were transfected with a full-length β_1A integrin-containing vector (pcDNA3.1/β_1A). Cells were lysed and protein extracts were blotted with anti-β_1A integrin antibody. β-Actin is the loading control. (**e**) Micrograph pictures of transfected cells after 48 hours of transfection. Bar = 50 μm. (**f**) HaCaT cells transfected with β_1B, β_1A, or empty vector were stained with TUNEL assay at 24 and 48 hours after transfection. (**g**) β_1B-transfected HaCaT cells were trypsinized 48 hours after transfection and kept in suspension in polypropylene tubes for different time points. Cells were lysed and lysates were blotted against anti-caspase-8 antibody. β-Actin was used as loading control. (**h**) Primary human keratinocytes were transfected with small interfering RNA (siRNA) β_1B or scramble and kept in suspension in polypropylene tubes for 12 hours. Cell lysates were blotted against anti-β_1B integrin or anti-caspase-8 antibody. β-Actin was used as loading control. (**i**) In the same experiment, cells were stained in propidium iodide (PI) solution. Sub-G1 peak positive cells (M1) were analyzed by flow cytometry. Data represent the mean from three independent experiments.

death, as it appeared from the significant increase in the percentage of TUNEL-positive cells at 24 and 48 hours after transfection with β_1B, but not with β_1A (Figure 6f). When keratinocytes were suspended, activation of caspase-8 at 0 hours occurred only in β_1B integrin-transfected cells. During suspension, activation of caspase-8 was observed earlier in β_1B integrin-overexpressing cells than in mock cells, as shown by the appearance of the active fragment at 3 and 6 hours (Figure 6g). On the other hand, caspase-8 activation was not detected in keratinocytes transfected with small interfering RNA (siRNA) β_1B at 12 hours (Figure 6h). Finally, the percentage of cells in sub-G1 peak at 12 hours is higher in scramble than in siRNA β_1B-transfected keratinocytes (Figure 6i).

DISCUSSION

In this study, we report that the β_1B isoform cooperates with antagonized β_1A integrin to induce a form of integrin-mediated cell death in human keratinocytes, independently of death receptors. Death domain-containing proteins trigger anoikis in epithelial cells (Frisch, 1999; Rytömaa *et al.*, 1999), and anoikis is mediated by Fas/FasL and by the activation of caspase-8 in endothelial cells (Aoudjit and Vuori, 2001). Furthermore, FADD has a critical role in detachment-induced apoptosis (Frisch, 1999), and epithelial cell lines expressing a dominant-negative form of FADD are protected from anoikis (Rytömaa *et al.*, 1999). In contrast, we show that although blocking β_1 integrin activates caspase-8, death receptors are not involved in this system. Indeed, both cell suspension and anti-β_1 integrin failed to upregulate Fas and FasL, and induced apoptosis in shFADD-infected keratinocytes, with an early activation of caspase-8. This is consistent with previous work showing that caspase-8 activation can occur in a manner independent of FADD (Stupack *et al.*, 2001). As keratinocytes undergo anoikis after the early activation of caspase-8, without the involvement of FADD, one might postulate that caspase-8 is activated downstream of caspase-3, in agreement with previous reports of death receptor-independent apoptosis (Ryu *et al.*, 2005). Alternatively, caspase-8 activation could occur downstream of caspase-9 as an amplification loop of the intrinsic apoptotic pathway (Grossmann *et al.*, 2001). However, both explanations have been ruled out by our previous study in which we reported that caspase-9 as well as executioner caspases are activated downstream of caspase-8 during keratinocyte anoikis (Marconi *et al.*, 2004).

Given the exclusion of death receptors from the apoptotic pathway, we pursued the hypothesis of a direct interaction between integrins and caspase-8. Indeed, this study provides evidence that β_1 integrin and caspase-8 colocalize in the cytoplasm and coimmunoprecipitate even before detachment. Immediately after suspension, caspase-8 is activated, whereas integrin is internalized and degraded. This suggests that keratinocyte suspension promotes β_1 integrin fragmentation of the extracellular domain. Interestingly, this fragment was previously shown to induce apoptosis through caspase-8 activation in myocytes (Menon *et al.*, 2006) and β_5 cytoplasmic domain is proapoptotic in SCC (squamous cell carcinoma) cell lines (Janes and Watt, 2004). Association of integrins with caspase-8 has been previously reported by Stupack *et al.* (2001) who, at variance with our study, observed a direct interaction of caspase-8 with β_3 integrin only in cell lines adherent to an inappropriate ligand. Furthermore, $\alpha_v\beta_3$ integrin has been recently reported to associate with caspase-8 in osteoclasts plated on a nonspecific matrix, and procaspase-8 was only detected in suspended cells (Zhao *et al.*, 2005). To our knowledge, this study describes a previously unreported form of IMD in normal human keratinocytes. However, at variance with the above publications, we show that a direct association between procaspase-8 and β_1 integrin occurs in keratinocytes still adherent to the proper ligand. Thus, in keratinocytes, β_1 integrin–procaspase-8 complex exists in a ''preapoptotic'' state until a stimulus triggers IMD, further confirming that integrins themselves can have a proapoptotic role (Giancotti and Ruoslahti, 1990; Plath *et al.*, 2000).

We also show that β_1B isoform has a critical role in IMD. β_1B integrin is mostly expressed in the cytoplasm and, unlike β_1 integrin, does not undergo degradation during suspension, whereas caspase-8 is activated. Moreover, β_1A and β_1B integrins cluster at the perinuclear level and immunoprecipitate with caspase-8 during suspension. A direct association between caspase-3 and $\alpha_5\beta_1$integrin was detected in non-adherent rat fibroblasts (Rajeswari and Pande, 2006) but, at variance with our study, activation of the caspase was observed only when the complex was translocated to the cell membrane, and not in the cytoplasm. Although the study did not address the signal allowing translocation, we hypothesize that in our system, the β_1B variant located in the cytoplasm cooperates with the cytoplasmic fragment of β_1A. This integrin fragment derives from the degradation of β_1A integrin, and proves the unligated state of this integrin. At the cytoplasmic level, the two integrin isoforms enhance the recruitment of caspase-8 and induce cell death, in good agreement with previous results on the proapoptotic role of integrins (Kozlova *et al.*, 2001).

The proapoptotic role of β_1B integrin was definitely shown both in cells overexpressing the complementary DNA and in cells in which expression of the isoform was silenced by β_1B siRNA. It seems that β_1B is responsible for keratinocyte apoptosis even before cell suspension, with the early activation of caspase-8. These results indicate that the expression of β_1B integrin is sufficient to induce IMD in human keratinocytes, even when cells are adherent to the appropriate ligand. We postulate that unligated β_1A integrin is internalized, recruits procaspase-8, and clusters with β_1B integrin that in turn is ligated to procaspase-8 at perinuclear level. Caspase-8 is then activated, leading to the initiation of the extrinsic apoptotic pathway.

SCCs are tumors that arise in multilayered epithelia such as the epidermis, esophagus, cervix, and the oral cavity. Although the tumors differ according to the tissue of origin in their frequency, etiology, and prognosis, they are all characterized by the alteration of integrins. In the epidermis, SCC may originate from keratinocyte stem cells (Zhang *et al.*, 2005), which are protected from anoikis by virtue of the high levels of β_1 integrin (Tiberio *et al.*, 2002). SCCs are

characterized by perturbation of integrin expression and extensive loss of the basement membrane (Savoia *et al.*, 1994; Ferreira *et al.*, 2009). Keratinocyte stem cells detach from the basement membrane, proliferate, and may reattach to an inadequate location to eventually metastasize. We speculate that β_1B integrin works as a ''sensor'' of the detachment from the proper ligand and stimulates apoptosis to prevent spread of SCC, thus acting as a ''fail-safe'' device.

MATERIALS AND METHODS

DNA constructs

Retroviral constructs pSUPER.Retro and pSUPER.Retro.shFADD were a kind gift of SM Frisch (West Virginia University, Morgantown, WV). A full-length β_1B or β_1A integrin complementary DNA were generated by reverse transcriptase-PCR and inserted in the pcDNA3.1/V5-His using the TOPO TA Expression kit (Invitrogen, Carlsbad, CA).

Cell culture and infection

Normal human keratinocytes were obtained from foreskin and cultured as described previously (Pincelli *et al.*, 1997). The spontaneously transformed keratinocyte line HaCaT was kindly provided by Dr N Fusenig (German Cancer Research Center (DKFZ), Heidelberg, Germany) and cultivated as already described (Boukamp *et al.*, 1988). Packaging lines for the described retroviral constructs were generated by transinfection in the ecotropic Phoenix and amphotropic GP + envAm12 cells, as previously described (Grande *et al.*, 1999). HaCat cells were transduced by two cycles of infection (6 hours each) with viral supernatant in the presence of polybrene (8 $\mu g\,ml^{-1}$). Transduced cells were selected in the presence of 1 $\mu g\,ml^{-1}$ puromycin (Sigma, St Louis, MO). Anoikis assay was performed as previously described (Marconi *et al.*, 2004).

Cell culture and transfection

HaCaT cells were transfected with described pcDNA3.1 plasmids with Lipofectamine 2000 and OptiMEM (Invitrogen) as suggested in the datasheet. Subconfluent normal human keratinocytes were double transfected with β_1B integrin siRNA (sense: 5′-CAAAGUGG CUUAUAAAGUAUU-3′; antisense: 5′-PUACUUUAUAAGCCA CUUUGUU-3′; Thermo Scientific, Dharmacon, Lafayette, CO) with Lipofectamine 2000 and OptiMEM (Invitrogen) as suggested in the datasheet. Protein levels were detected by western blotting as described below. Anoikis assay was performed as previously described (Marconi *et al.*, 2004).

Antibodies

Rabbit polyclonal antibodies used were: anti-caspase 8 (AR-18; gift of JC Reed, The Burnham Institute, La Jolla, CA); anti-β_1 integrin (M-106; Santa Cruz Biotechnology, Santa Cruz, CA); anti-β_1A and anti-β_1B antisera (gift from G Tarone, University of Turin, Turin, Italy). Mouse monoclonal antibodies used were: neutralizing anti-β_1 integrin (Lia 1/2; Immunotech, Marseile, France); anti-β_1 integrin (K20; Santa Cruz Biotechnology); anti-caspase 8 (Ab-3; Calbiochem, Darmstadt, Germany); anti-active caspase 8 (C15; Enzo Life Sciences, Farmingdale, NY); anti-FADD (A66-2; BD Biosciences, San Jose, CA); and anti-β actin (AC-15; Sigma). Secondary antibodies, Alexa Fluor 546 and 488-conjugated goat IgGs (Invitrogen) and horseradish peroxidase-conjugated goat IgGs, were also used (Bio-Rad, Hercules, CA).

FACS and flow cytometry

For anoikis (sub-G1 peak positive), cells were suspended in hypotonic fluorochrome solution (propidium iodide 50 $mg\,ml^{-1}$, 0.1% sodium citrate, and 0.1% Triton X-100), labeled at 4 °C for at least 15 minutes, and then analyzed using Epics XL flow cytometer (Epics XL, Coulter, Hialeah, FL). Plots were analyzed using WinMDI 2.8 software (http://facs.scripps.edu/software.html).

Both cells that had undergone anoikis or treated with pemphigus vulgaris immunoglobulin G, as a positive control for FasL expression, were analyzed for extracellular antigen expression. Cells were labeled with monoclonal anti-human Fas antibody (clone UB2; Immunotech) and with biotin-conjugated rabbit anti-mouse immunoglobulin (Dako, Copenaghen, Denmark), or with biotin-conjugated anti-human FasL monoclonal antibody (Clone NOK-1; BD PharMingen) diluted in phosphate-buffered saline with 5% fetal bovine serum. Cells were then labeled with streptavidin/RPE-Cy5 (Dako) and analyzed using Epics XL flow cytometer (Coulter). Plots were analyzed using WinMDI 2.8 software.

ELISA

To measure the release of soluble FasL during anoikis, we performed a two-site ELISA (MBL, Nagoya, Japan) on culture media according to the manufacturer's instructions.

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed in selected infected keratinocytes. After treatment with tumor necrosis factor-related apoptosis-inducing ligand (kindly provided by Dr Martin Leverkus, Magdeburg, Germany) and cycloheximide 1 $\mu g\,ml^{-1}$, cells were incubated with MTT (Sigma) as previously described (Truzzi *et al.*, 2008). Results were expressed as the mean ± SD of three different experiments. The Student's *t*-test was used for comparison of the means.

Coimmunoprecipitation and immunoblotting

Lysates were processed for propidium iodide and western blotting as described previously (Marconi *et al.*, 2004) or with lysis buffer (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1% deoxycolate, 1% Triton X-100, 0.1% SDS, 0.2% sodium azide, and inhibitor cocktail (Roche Diagnostics, Basel, Switzerland)). Proteins were immunoprecipitated with anti-β_1 integrin (K20) or anti-β_1 antisera. Samples were resolved by 7 or 12% SDS-PAGE and immunoblotted as described previously (Marconi *et al.*, 2004).

Immunofluorescence analysis

Subconfluent cells were fixed in ice-cold methanol or 4% paraformaldehyde and incubated with monoclonal anti-β_1 integrin antibody and the appropriate secondary antibody. Cells were then labeled with polyclonal anti-caspase 8 antibody and appropriate secondary antibody. Alternatively, cells were first stained with rabbit anti-β_1 integrin variant antisera and with mouse anti-active caspase-8. Micrographs were taken on a Confocal Scanning Laser Microscopy (Leica TCS4D; Leica, Exton, PA). In selected cases, a series of images of the same *x–y* plane was taken and the reconstruction made on the *z*-axis.

Internalization assay

Keratinocytes were preincubated with neutralizing anti-β_1 integrin antibody and anoikis assay was performed. At each time point, cells

were fixed in 4% paraformaldehyde and immunofluorescence performed with the addition of the secondary antibody.

Reverse transcriptase-PCR

Total RNA was extracted from transfected cells and reverse transcriptase-PCR was performed as previously described (Truzzi *et al.*, 2008). Nucleotide sequences of the β_1B integrin oligomers used (MWG Biotech, Ebersberg, Germany) were: β_1B-DP 5′-GGGA ACAACGAGGTCATGGTTCAT-3′ and β_1B-RP 5′-TTATAAGCCACT TTGCTTTTTGGATG-3′.

TUNEL assay

Cells were fixed in 4% paraformaldehyde both 24 and 48 hours after transfection. Cells were then stained with the *In Situ* Cell Death Detection Kit (Roche) as recommended by the manufacturer. Positive cells were analyzed as detailed previously (Truzzi *et al.*, 2008).

CONFLICT OF INTEREST

The authors state no conflict of interest.

ACKNOWLEDGMENTS

We thank Professor G Tarone for the critical reading of this paper. This work was partially supported by a grant from the Ministry of Instruction, University, and Research (20077FPJ22).

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

REFERENCES

Altruda F, Cervella P, Tarone G *et al.* (1990) A human integrin beta 1 subunit with a unique cytoplasmic domain generated by alternative mRNA processing. *Gene* 95:261–6

Aoudjit F, Vuori K (2001) Matrix attachment regulates Fas-induced apoptosis in endothelial cells: a role for c-flip and implications for anoikis. *J Cell Biol* 152:633–43

Arredondo J, Chernyavsky AI, Karaouni A *et al.* (2005) Novel mechanisms of target cell death and survival and of therapeutic action of IVIg in Pemphigus. *Am J Pathol* 167:1531–44

Balzac F, Belkin AM, Koteliansky VE *et al.* (1993) Expression and functional analysis of a cytoplasmic domain variant of the beta 1 integrin subunit. *J Cell Biol* 121:171–8

Boukamp P, Petrussevska RT, Breitkreutz D *et al.* (1988) Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106:761–71

Brassard DL, Maxwell E, Malkowski M *et al.* (1999) Integrin alpha(v)beta(3)-mediated activation of apoptosis. *Exp Cell Res* 251:33–45

Ferreira M, Fujiwara H, Morita K *et al.* (2009) An activating beta1 integrin mutation increases the conversion of benign to malignant skin tumors. *Cancer Res* 69:1334–42

Fleischmajer R, Perlish JS, MacDonald ED *et al.* (1998) There is binding of collagen IV to beta 1 integrin during early skin basement membrane assembly. *Ann NY Acad Sci* 857:212–27

Frisch SM (1999) Evidence for a function of death-receptor-related, death-domain-containing proteins in anoikis. *Curr Biol* 9:1047–9

Frisch SM, Francis H (1994) Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 124:619–26

Giancotti FG, Ruoslahti E (1990) Elevated levels of the alpha 5 beta 1 fibronectin receptor suppress the transformed phenotype of Chinese hamster ovary cells. *Cell* 60:849–59

Grande A, Piovani B, Aiuti A *et al.* (1999) Transcriptional targeting of retroviral vectors to the erythroblastic progeny of transduced hematopoietic stem cells. *Blood* 93:3276–85

Grossmann J, Walther K, Artinger M *et al.* (2001) Apoptotic signaling during initiation of detachment-induced apoptosis (''anoikis'') of primary human intestinal epithelial cells. *Cell Growth Differ* 12:147–55

Howlett AR, Bailey N, Damsky C *et al.* (1995) Cellular growth and survival are mediated by beta 1 integrins in normal human breast epithelium but not in breast carcinoma. *J Cell Sci* 108:1945–57

Janes SM, Watt FM (2004) Switch from alphavbeta5 to alphavbeta6 integrin expression protects squamous cell carcinomas from anoikis. *J Cell Biol* 166:419–31

Kee WJ, Li ER, Watt FM (2000) beta1B integrin subunit contains a double lysine motif that can cause accumulation within the endoplasmic reticulum. *J Cell Biochem* 78:97–111

Kozlova NI, Morozevich GE, Chubukina AN *et al.* (2001) Integrin alphavbeta3 promotes anchorage-dependent apoptosis in human intestinal carcinoma cells. *Oncogene* 20:4710–7

Marconi A, Atzei P, Panza C *et al.* (2004) FLICE/caspase-8 activation triggers anoikis induced by beta1-integrin blockade in human keratinocytes. *J Cell Sci* 117:5815–23

Menon B, Krishnamurthy P, Kaverina E *et al.* (2006) Expression of the cytoplasmic domain of beta1 integrin induces apoptosis in adult rat ventricular myocytes (ARVM) via the involvement of caspase-8 and mitochondrial death pathway. *Basic Res Cardiol* 101:485–93

Muzio M, Stockwell BR, Stennicke HR *et al.* (1998) An induced proximity model for caspase-8 activation. *J Biol Chem* 273:2926–30

Pincelli C, Haake AR, Benassi L *et al.* (1997) Autocrine nerve growth factor protects human keratinocytes from apoptosis through its high affinity receptor (TRK): a role for BCL-2. *J Invest Dermatol* 109:757–64

Plath T, Detjen K, Welzel M *et al.* (2000) A novel function for the tumor suppressor p16(INK4a): induction of anoikis via upregulation of the alpha(5)beta(1) fibronectin receptor. *J Cell Biol* 150:1467–78

Rajeswari J, Pande G (2006) Direct association between caspase 3 and alpha5beta1 integrin and its role during anoikis of rat fibroblasts. *Cell Biol Int* 30:963–9

Retta SF, Balzac F, Ferraris P *et al.* (1998) beta1-integrin cytoplasmic subdomains involved in dominant negative function. *Mol Biol Cell* 9:715–31

Rytömaa M, Martins LM, Downward J (1999) Involvement of FADD and caspase-8 signalling in detachment-induced apoptosis. *Curr Biol* 9:1043–6

Ryu HY, Emberley JK, Schlezinger JJ *et al.* (2005) Environmental chemical-induced bone marrow B cell apoptosis: death receptor-independent activation of a caspase-3 to caspase-8 pathway. *Mol Pharmacol* 68:1087–96

Savoia P, Cremona O, Trusolino L *et al.* (1994) Integrins and basement membrane proteins in skin carcinomas. *Pathol Res Pract* 190:950–4

Stupack DG, Puente XS, Boutsaboualoy S *et al.* (2001) Apoptosis of adherent cells by recruitment of caspase-8 to unligated integrins. *J Cell Biol* 155:459–70

Tiberio R, Marconi A, Fila C *et al.* (2002) Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. *FEBS Lett* 524:139–44

Truzzi F, Marconi A, Lotti R *et al.* (2008) Neurotrophins and their receptors stimulate melanoma cell proliferation and migration. *J Invest Dermatol* 128:2031–40

Zhang W, Hanks AN, Boucher K *et al.* (2005) UVB-induced apoptosis drives clonal expansion during skin tumor development. *Carcinogenesis* 26:249–57

Zhao H, Ross FP, Teitelbaum SL (2005) Unoccupied alpha(v)beta3 integrin regulates osteoclast apoptosis by transmitting a positive death signal. *Mol Endocrinol* 19:771–80